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PIF1: a DNA helicase in yeast mitochondria

Andrée Lahaye, Hans Stahl[1], Denyse Thines-Sempoux and Françoise Foury

Unité de Biochimie Physiologique, Université Catholique de Louvain, Place Croix du Sud 2 B20, 1348 Louvain-La-Neuve, Belgium and [1]Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, FRG

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The *PIF1* gene is involved in repair and recombination of mitochondrial DNA (mtDNA). In this study, the *PIF1* gene product, which cannot be identified in normal yeast cells, has been overproduced from the GALI promoter to detectable protein levels. Location of PIF1 in mitochondria has been shown by immunoelectron microscopy and *in vivo* import experiments using ts *mas1* mutants deficient in the mitochondrial matrix-localized processing protease. Overproduction of PIF1 protein in *pif1* mutants restores mtDNA recombination proficiency but is toxic to yeast cells as observed by slower growth. The overproduced PIF1 protein, which is firmly associated with insoluble mitochondrial structures, has been partially purified in a mitochondrial nuclease deficient *nuc1* strain by a procedure including solubilization by urea and renaturation by dialysis at alkaline pH. PIF1 is a single-stranded (ss) DNA-dependent ATPase and a DNA helicase which unwinds partially DNA duplexes in a 5′ to 3′ direction with respect to the ss DNA on which it binds first.

Key words: gene overexpression/helicase/mitochondria/recombination and repair/yeast

Introduction

The mitochondrial genome is a multiple-copy system whose evolution has been rather independent from that of the nucleus. Therefore, identification and biochemical characterization of gene products involved in mtDNA metabolism is of key importance to understand the specific mechanisms of the replication, recombination and repair pathways. In *Saccharomyces cerevisiae*, even though several mitochondrially-encoded site-specific endonucleases with transposase/recombinase function have been characterized (Kotylak *et al.*, 1985; Dujon, 1989; Perlman and Butow, 1989), the large majority of the proteins involved in mtDNA metabolism is encoded in the nucleus. To date, no general view of mtDNA metabolism has emerged.

Several years ago, we have identified in *S.cerevisiae* a nuclear gene called *PIF1* whose product is required for the repair of mtDNA after UV light and ethidium bromide treatments (Foury and Kolodynski, 1983) and for the maintenance of mtDNA at elevated temperature (Foury, 1990). Moreover, the *PIF1* gene product promotes high recombination frequency between rho$^+$ and tandemly-arrayed rho$^-$ genomes in diploids through a process involving the recognition of specific recombinogenic signals in the mtDNA (Foury and Kolodynski, 1983; Foury and Van Dyck, 1985). Genetic and sequencing data (Foury and Lahaye, 1987) have shown that it encodes a potential mitochondrial protein of 857 amino acids and calculated M_r = 97 500. The seven consensus sequences which characterize a super-family of DNA helicases in *Escherichia coli* and plant/animal viruses are also present in the PIF1 protein (Hodgman, 1988; Gorbalenya *et al.*, 1988, 1989).

The aim of this work was to determine by biochemical methods the enzymatic activities of the PIF1 protein. To date, no DNA helicase has ever been detected in mitochondria and our approach was hampered initially by the difficulty of identifying the PIF1 polypeptide in a yeast extract. In this study, we report the overproduction, mitochondrial location and biochemical characterization of the partially purified PIF1 protein as a ss DNA-dependent ATPase and DNA helicase which unwinds partially duplex DNA in a 5′ to 3′ polarity.

Results

As the number of genes cloned by restoration of the wild type phenotype in mutants transformed with a multi-copy plasmid library increases, it is becoming more and more evident that suppressors can be cloned instead of the authentic gene. However, the wild type phenotype is rarely restored by centromeric vector-borne suppressor genes and the disruption of the suppressor genes rarely leads to deficiency phenotypes identical to those observed in the original mutants. Our cloned gene restores wild type PIF1 phenotype when placed in a centromeric vector (unpublished) and its chromosomal disruption elicits the same pleitropic deficiencies as those observed in *pif1* mutants. In tetrads issued from a cross between wild type and disrupted strains, there is a perfect cosegregation of the disrupted gene and deficiency phenotypes, indicating that the latter are the consequence of the disruption and are not artefactual. Moreover, we have shown that in diploids issued from crosses between a strain bearing a disrupted allele of the cloned gene and *pif1-1* or *pif1-2* mutants, no restoration of the wild type phenotype is observed. Since the *pif1* and null mutants are recessive, the *PIF1* and disrupted genes are allelic and, therefore, it can be definitively concluded that we have cloned the authentic *PIF1* gene, as previously published (Foury and Lahaye, 1987).

Overexpression and detection of PIF1 protein in yeast.

To facilitate the purification of the PIF1 protein, we overexpressed the *PIF1* gene in yeast using the inducible GALI promoter. As shown in Figure 1, we used a 2 μm-based multicopy vector bearing the GALI promoter, and *URA3* and *LEU2D* as selection markers. The *PIF1* gene,

flanked by its non coding sequences, which include 80 bp upstream of the *PIF1* AUG initiation codon and the 3′ terminator region, was inserted into the vector downstream of the GALI promoter.

In order to identify the PIF1 protein unambiguously in yeast, immunodetection experiments were carried out by using goat polyclonal antibodies raised against a PIF1-β-galactosidase fusion protein produced in *E.coli* (see Materials and methods). After galactose induction, a novel major polypeptide of the expected molecular weight (~95 kd) was detected by polyacrylamide gel electrophoresis in mitochondrial extracts of the PIF1 overproducing strain (Figure 2A, lane f) in contrast to mitochondrial extracts from the null *pif1* mutant and the wild type strain containing the control vector pLGALZ3 without the *PIF1* insert (Figure 2A). The overproduced PIF1 protein was visualized by immunodetection (Figure 2B) and appeared as three polypeptides of about 95, 97 and 100 kd respectively, with the 100 kd species assumed to be the mitochondrial precursor (see below). The two other polypeptides are rather diffuse and the heterogeneity is thought to result from mitochondrial proteolysis or post-translational modification. In the latter case, it would not be surprising that an excess of PIF1 in the mitochondria leads to the accumulation of unmodified proteins. Post-mitochondrial supernatants of the disrupted and the wild type strain showed no cross-reaction with the anti-PIF1 antibodies (lanes a and b). The very weak detection found in the post-mitochondrial fraction of the overproducing strain (lane c) is not in contradiction with the mitochondrial

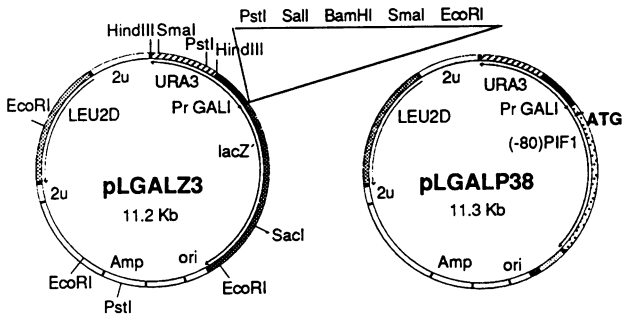

Fig. 1. Yeast expression vectors with GALI promoter. pLGALZ3 (no insert) and pLGALP38 (*PIF1* insert). Details of plasmid construction will be published elsewhere. Only relevant restriction sites are indicated.

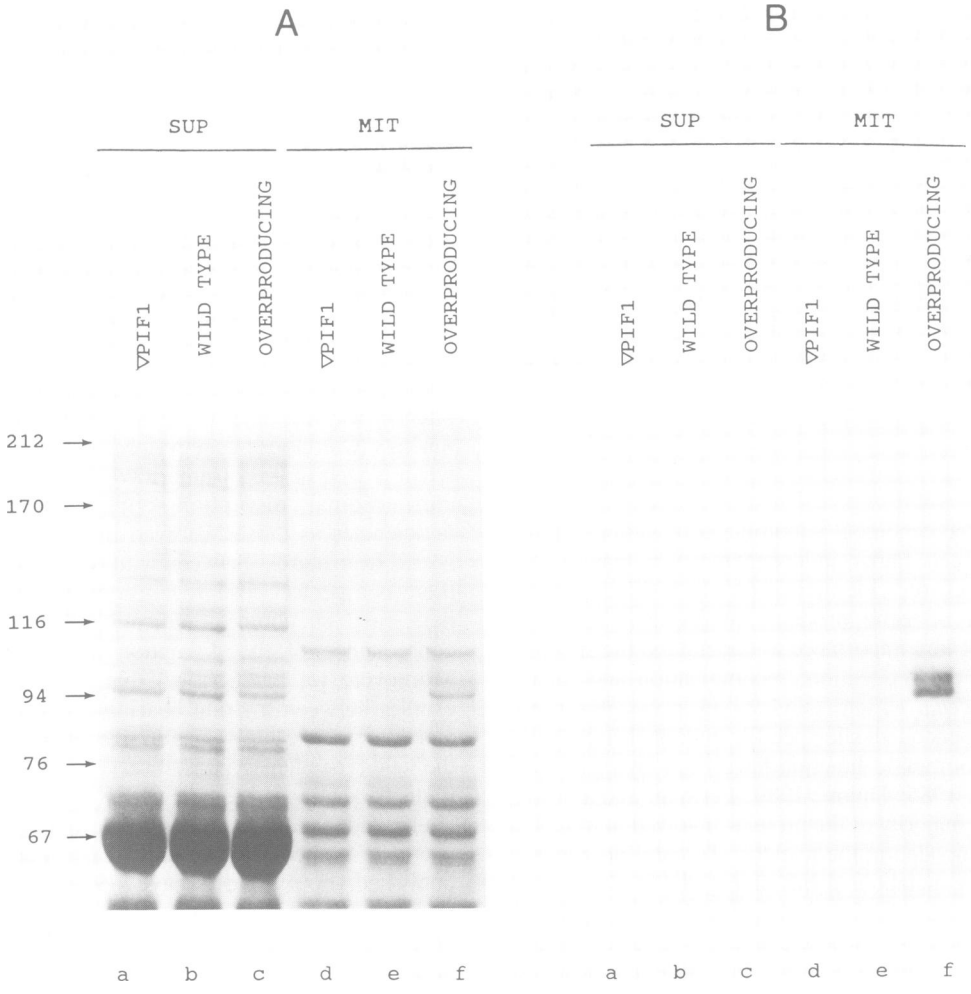

Fig. 2. Overexpression of the *PIF1* gene in *S.cerevisiae*. A and B, mitochondria (lanes d–f) and post-mitochondrial supernatants (lane a–c) were isolated from galactose grown cells as described in Materials and methods. Proteins were separated by electrophoresis in an 8% SDS–polyacrylamide gel and stained with Coomassie blue (**A**, 100 μg protein) or subjected to immunodetection with anti-PIF1 antibodies and [^{35}S]protein A after transfer to nitrocellulose (**B**, 25 μg protein). Lanes a and d, null *pif1* mutant A25/PGD; lane b and e, strain A25/G3 carrying the control vector pLGALZ3; lanes c and f, strain A25/P38 carrying the *PIF1* overexpressing plasmid pLGALP38.

location of PIF1 as the isolation of the mitochondrial pellet is only based on a differential centrifugation procedure. The location of PIF1 protein in mitochondria was verified in further experiments (see below). Western blot analysis showed that no polypeptide was detected by anti-PIF1 antibodies in samples containing the mitochondrial extracts from the *pif1* disrupted strain (lane d) or from the galactose grown wild type strain transformed by the control vector pLGALZ3 (lane e). The absence of signal in wild type extracts probably reflects the low abundance of the PIF1 product in yeast mitochondria as predicted by the low transcription level and by the codon bias index which is close to zero and negative. Similar difficulty in identifying in wild type strains proteins involved in DNA metabolism was reported for the poorly expressed mitochondrial CBP1 (Weber and Dieckmann, 1990) and nuclear RAD3 (Sung *et al.*, 1987a) gene products.

In vivo effects of the overproduced PIF1 protein on mtDNA recombination and cell growth

The overproduced PIF1 protein was shown to be active by its ability to complement mtDNA recombination deficiency in *pif1* mutants. The *PIF1* gene is required for the recombination of mtDNA in crosses between rho$^+$ and tandem PIF-dependent rho$^-$ strains (Foury and Kolodynski, 1983) and the recombination test consists in the selection of those rho$^+$ diploids that have integrated in their mtDNA a drug resistance marker initially borne by the rho$^-$ genome. When the frequency of recombination is high among the diploids, a confluent growth is observed on a solid medium containing the drug and glycerol as the non-fermentable carbon source. In *pif1* mutants, no growth or only papillae are observed.

In order to demonstrate that the overproduced PIF1 protein is biologically active, we verified that recombination proficiency was restored in a null mutant after transformation with the vector pLGALP38 and galactose induction. Figure 3B (panel b, lane I) shows the confluent growth of oligomycin resistant diploids resulting from the cross on solid galactose medium (YGAL) of the *PIF1* overexpressing transformants with the rho$^-$ mutant JF2-2B/1-41 which carries the mitochondrial O^R_{625} allele. In contrast, no restoration of the recombination was observed with transformants harboring the *PIF1* insertless vector (pLGALZ3) (lane II) or when the *PIF1* overexpressing transformants were crossed with the rho$^-$ strain on a non-

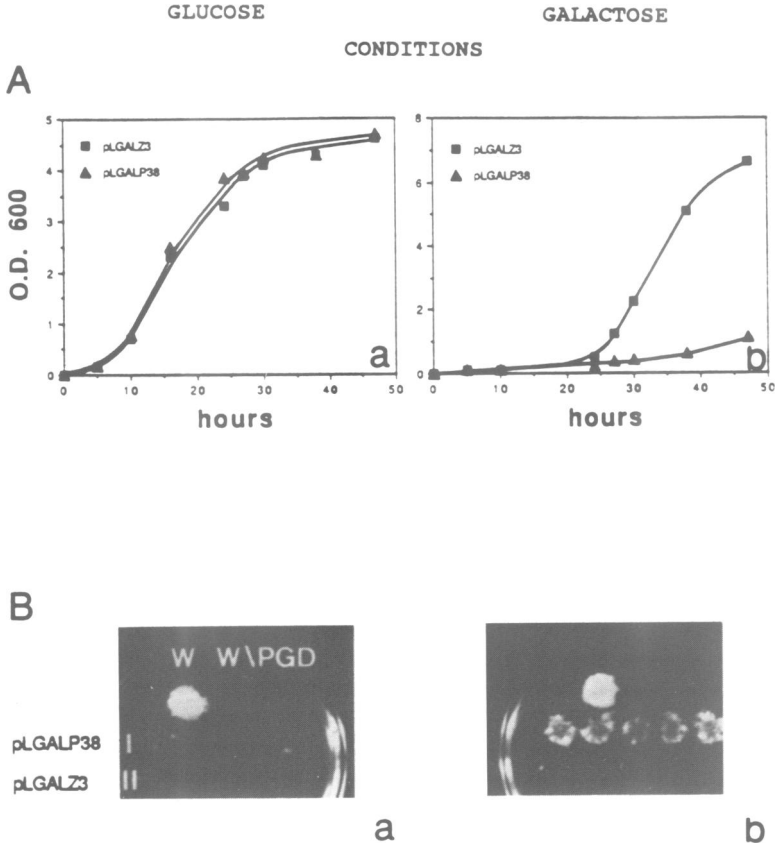

Fig. 3. *In vivo* effects of the overproduced PIF1 protein on cell growth and mtDNA recombination. Toxicity of *PIF1* overexpression of the *PIF1* is shown in **A**. Wild type strain A25/G3 carrying the control vector (closed squares) or the PIF1 overproducing strain A25/P38 (closed triangles) were grown in SD (graph a) or SGAL (graph b) media after inoculation to an optical density of ~0.05 with cells freshly grown in a SETOH medium. Cell growth was measured by optical density at 600 nm. Restoration of PIF-dependent recombination proficiency in *pif1* mutants by the overproduced PIF1 protein is shown in B. The null *pif1* mutant W303-1B/PGD was transformed with the control vector pLGALZ3 (II) or the *PIF1* overexpressing vector pLGALP38 (I). The transformants were crossed with the *pif1* rho$^-$ O^R_{625} mutant JF2-2B/1-41 on YGAL galactose (panel b) or YD glucose (panel a) solid media. After incubation at 30°C for 24 h, the diploids were replicated and selectively grown for 24 h on glucose minimal medium (SD). Replica-plating on solid medium containing glycerol plus oligomycin (see Materials and methods) selected those rho$^+$ diploids which have integrated the O^R_{625} allele in their mtDNA. Mother strains W303-1B (W) and W303-1B/PGD (W/PGD) were used as positive and negative controls.

inducible glucose medium (YD) (panel a, lane I). Negative and positive controls were obtained by crossing respectively the null *pif1* W303-1B/PGD mutant (W/PGD) and the wild type W303-1B strain (W) with JF2-2B/1-41.

Overproduction of the PIF1 protein was however toxic to yeast cells by impairing cell growth. This was first suggested by the impossibility of isolating viable transformants upon transformation with a *PIF1* gene constitutively overexpressed under the control of the ADC1 promoter and using the *LEU2D* gene as the selection marker (not shown). When the *PIF1* gene was under the control of the inducible GAL1 promoter, a slower growth of the transformants was observed upon induction conditions compared to a strain carrying the pLGALZ3 vector without insert (Figure 3A, graph b) or to the same transformants incubated in a non-inducible glucose minimal medium (graph a). Toxicity was not associated with defective mitochondrial DNA metabolism since no increased rho$^-$ production was observed during galactose induction. Normal growth was restored when the *PIF1* gene was altered by creating either a chimeric *PIF1-lacZ* gene which has only conserved the first 1000 bp of the *PIF1* gene or a frameshift mutation by deleting two internal closely linked *Bam*HI sites of the *PIF1* gene (not shown). These results suggest that the toxicity results from overexpression of the active PIF1 protein.

Localization of PIF1 protein in yeast mitochondria

Several lines of evidence indicate that the PIF1 protein is located in mitochondria. First, as previously reported (Foury and Lahaye, 1987), the sequence of the N-terminal region of the PIF1 protein exhibits the typical features of mitochondria-targeting presequences. Second, recombination/repair deficiency phenotypes in *pif1* mutants have only been observed at the mtDNA level. Third, analysis of the mitochondrial and post-mitochondrial fractions obtained by differential centrifugation have shown that the overproduced PIF1 protein is associated with mitochondria. Fourth, the overproduced PIF1 protein restores the mtDNA recombination proficiency in *pif1* mutants.

However, to rule out any indirect effect, we verified that the PIF1 protein is imported into mitochondria as follows. If the PIF1 protein is located in mitochondria, its import should be impaired in *mas1* mutants (Yaffe and Schatz, 1984). Yeast strains carrying a thermosensitive *mas1* mutation are deficient in the matrix-localized protease responsible for the cleavage and the import of mitochondrial precursors. At the non-permissive temperature, the precursors accumulate in the cell. Therefore, at 37°C ts *mas1* strains transformed by the *PIF1* overexpressing plasmid should accumulate the PIF1 mitochondrial precursor under galactose induction. We indeed observed an increase in molecular weight of the overproduced PIF1 protein at 37°C (Figure 4, lane d). In contrast, only the lower molecular weight forms were present in the *MAS1* isogenic strain (lanes a and b). We concluded that the higher molecular weight form accumulating in the *mas1* mutant is the PIF1 precursor which otherwise is matured in a lower molecular weight form by the mitochondrial matrix-localized *MAS1*-encoded protease.

Mitochondrial location of the PIF1 protein was further confirmed by immunoelectron microscopy (IEM) using purified anti-PIF1 immunoglobulins (see Materials and methods). Gold particles were found only in the mitochondria

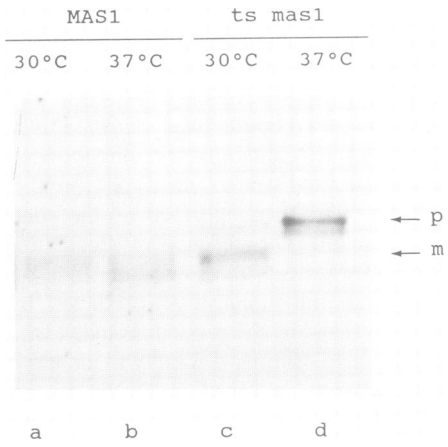

Fig. 4. PIF1 mitochondrial precursor accumulates in *mas1* mutants. The wild type strain AH216-1A and the isogenic ts *mas1* mutant MY11-D72 were transformed by the *PIF1* overexpressing plasmid pLGALP38 (respectively named AH216-1A/P38 and MY11/P38). Expression of the *PIF1* gene was induced by galactose as described in Materials and methods, except that, for the expression at the non-permissive temperature, cells were incubated at 37°C for 3 h before addition of galactose. Total yeast proteins (100 μg) of AH216-1A/P38 (lane a, induction of PIF1 at 30°C; lane b, induction at 37°C) and MY11/P38 (lane c, induction at 30°C; lane d, induction at 37°C) were separated by electrophoresis in an 8% SDS−polyacrylamide gel, transferred to nitrocellulose, and subjected to immunodetection with anti-PIF1 antibodies and [^{35}S]protein A. p = precursor; m = mature.

of the PIF1 overproducing strain and more especially in 'black bodies' that are specifically present here (Figure 5, panels A and D). These 'bodies' might be the so-called 'inclusion bodies' that are often observed in strains artificially overproducing a protein (reviewed by Martson and Hartley, 1990). No gold particles were observed in the null *pif1* mutant (panel B) and in the non-overexpressing wild type strain (panel C) treated with anti-PIF1 IgG, or in PIF1 overproducing cells incubated with preimmune purified IgG (panel A). Immunoelectron microscopy therefore confirmed that the PIF1 protein is targeted to the mitochondria.

Partial purification of the PIF1 protein

Sequence analysis of the *PIF1* gene has shown that the PIF1 polypeptide possesses the seven consensus sequences present in a large family of DNA helicases (Hodgman, 1988; Gorbalenya *et al.*, 1988, 1989; Foury, 1990; Seraphin, 1990). DNA helicases are enzymes which melt the hydrogen bonds of DNA duplexes. They have an associated ss nucleic acid-dependent NTPase activity which is presumed to provide the energy required for DNA unwinding (reviewed by Matson and Kaiser-Rogers, 1990; Thömmes and Hübscher, 1990).

For *in vitro* DNA helicase assays, we have partially purified the PIF1 protein overproduced in a yeast strain devoid of the extremely active mitochondrial NUC1 exo-endonuclease (Zassenhaus *et al.*, 1988). Osmotic shock of mitochondria and treatment of mitochondrial membranes with non ionic detergents failed to release the PIF1 protein into the soluble fraction. Zwitterionic tensides such as zwittergent 3-12 and lysolecithine in combination with salt or denaturant agents were solubilizing the PIF1 protein, resulting, however, in enzymatic inactive extracts. It has been shown in several cases that highly insoluble proteins

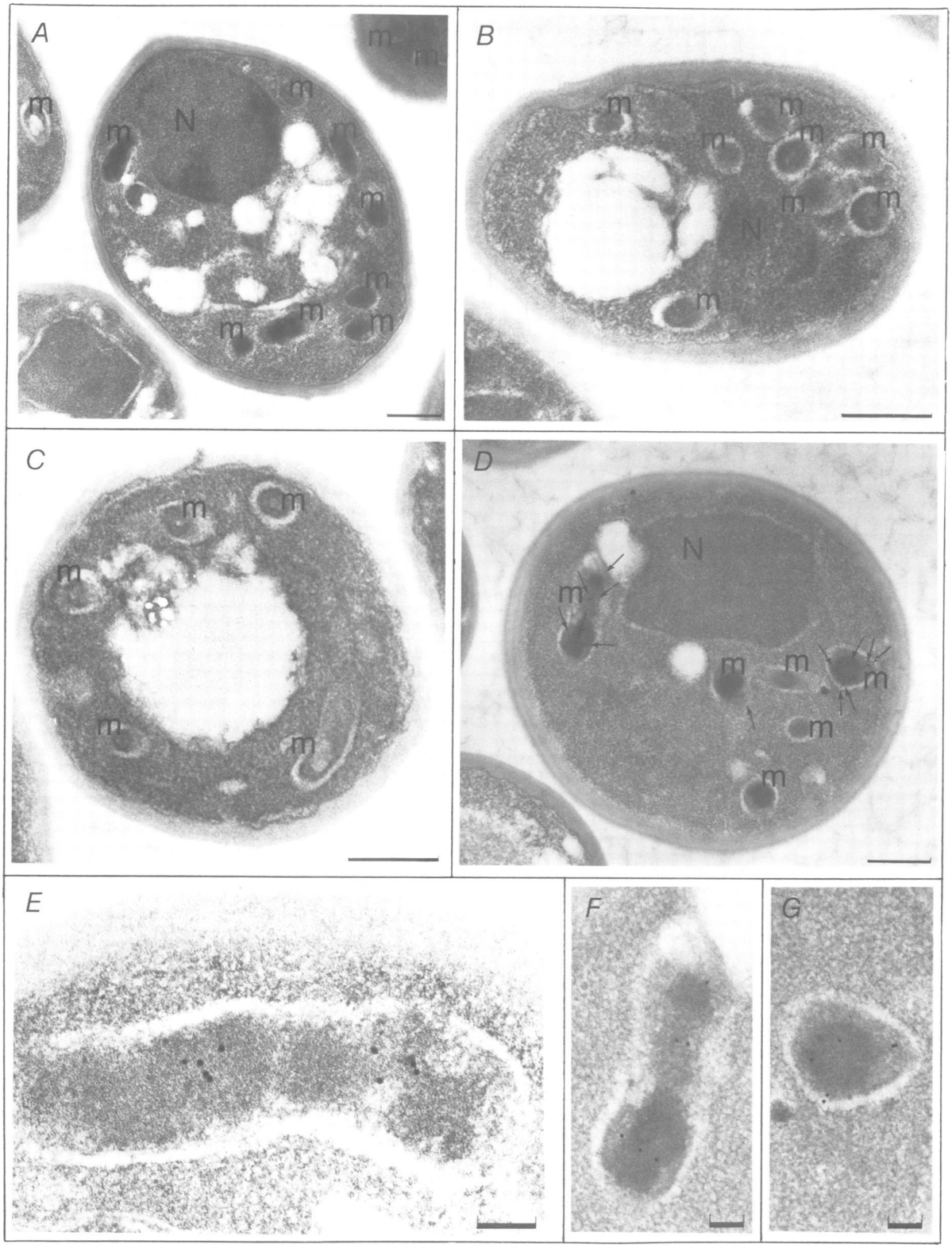

Fig. 5. Subcellular localization of PIF1 protein by immunogold labeling on thin sections of yeast cells. Cells grown in galactose were prepared for immunoelectron microscopy (IEM) experiments and were immunogold labeled as described in Materials and methods. Thin sections performed at room temperature of the *PIF1* overexpressing A25/P38 (**D**), null *pif1* mutant A25/PGD (**B**) and the non-overexpressing wild type A25/G3 (**C**) cells were treated with purified anti-PIF1 immunoglobulins (IgG). In panel **A**, A25/P38 cells were treated with preimmune IgG. In panel **D**, small arrows indicate gold particles which are magnified in panels **F** and **G**. Another magnified mitochondrion of the overexpressing transformant is shown in panel E. m = mitochondria; N = nucleus. Black bars represent 0.5 μm for panels A, B, C and D and 0.1 μm for panels E, F and G.

could be solubilized by using a 'denaturation–renaturation' procedure with urea or guanidine as denaturant agents (reviewed by Martson and Hartley, 1990). We took advantage of this procedure and elaborated a protocol to solubilize and partially purify the PIF1 protein (see Materials and methods). After a stripping of mitochondrial membranes

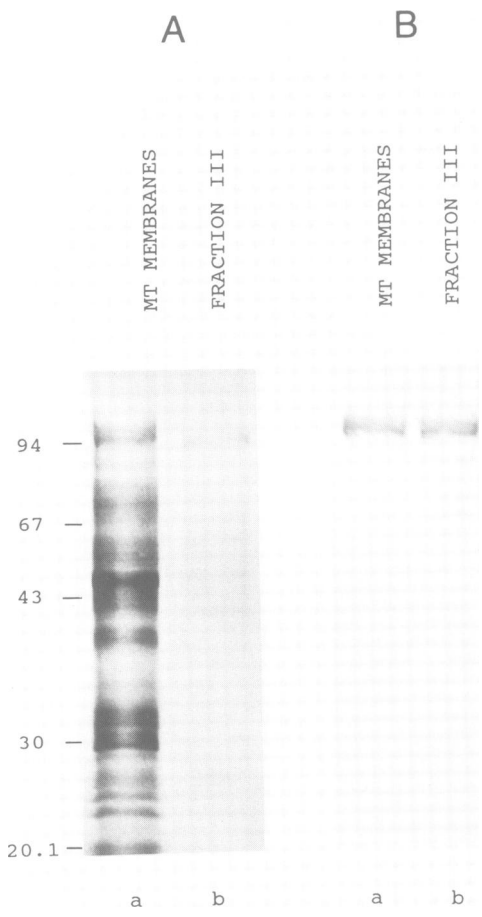

Fig. 6. Partial purification of PIF1 protein. Mitochondrial membranous proteins (lane a, 100 μg of proteins) and fraction III (lane b, 5 μg of proteins) were obtained from the PIF1 overproducing strain A25/P38 as described in Materials and methods. Proteins were separated by electrophoresis in a 10% SDS–polyacrylamide gel and stained with Coomassie blue (**A**) or subjected to immunodetection after transfer to nitrocellulose and treatment with anti-PIF1 antibodies and [^{35}S]protein A (**B**, one quarter of the protein quantity used for Coomassie blue staining).

by Triton X-100, the PIF1 protein was solubilized in 6.5 M urea, further purified on hydroxylapatite and renatured by dialysis. Renaturation by dialysis required an alkaline pH (pH 9.0) to avoid precipitation of the PIF1 protein and presence of β-mercaptoethanol (β-MSH) was necessary to obtain an active protein. Figure 6 shows that the PIF1 protein is significantly enriched in fraction III (lane b) compared to the mitochondrial membrane fraction (lane a).

PIF1 protein is a DNA-dependent ATPase

Trying to determine whether a DNA-dependent ATPase activity was associated with the PIF1 protein, we assayed fraction III from the PIF1 overproducing strain and from an isogenic strain harboring a disrupted copy of the *PIF1* gene.

Using the homopolynucleotide poly(dC) as a cofactor, a ss DNA-dependent ATPase activity was found in the extract from the overproducing strain. The specific activity in fraction III was 15 units/mg of proteins (one unit of activity is defined as the amount of enzyme that hydrolyzes 1 μmol of ATP/min at 37°C). ATPase activity assayed without

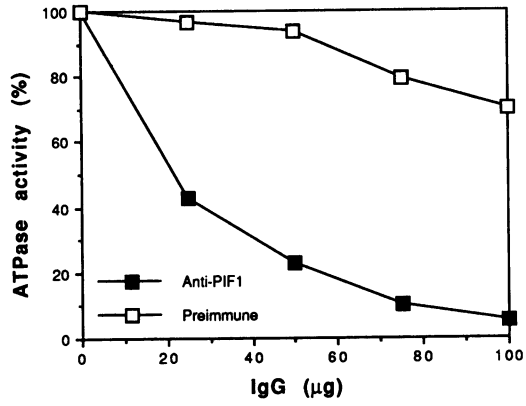

Fig. 7. Inhibition of the DNA-dependent ATPase activity by anti-PIF1 antibodies. Fraction III containing the PIF1 protein was incubated with increasing amounts of purified anti-PIF1 (closed squares) or preimmune (open squares) IgG at 4°C for 1 h. The ATPase reaction mixture was then added (final reaction volume = 50 μl) to start the reaction at 37°C (see Materials and methods). DNA-dependent ATPase activity is expressed as a percentage of the activity obtained in absence of immmunoglobulins (referred as 100%).

poly(dC) was negligible. In contrast, no activity was found in the extract from the *pif1* disrupted strain.

Evidence that PIF1 protein possesses a DNA-dependent ATPase activity was further confirmed by antibody inhibition studies. Increasing amounts of purified anti-PIF1 IgG progressively inhibited the ATPase activity in extracts from the PIF1 overproducing strain while no inhibition was observed with purified preimmune IgG (Figure 7).

PIF1 protein is a DNA helicase

DNA helicase activity was determined by the capacity of the PIF1 protein to unwind a partially duplex DNA consisting of a circular ss M13 DNA annealed to a complementary 41-mer elongated oligonucleotide (see Materials and methods). The fraction III from the null *pif1* mutant had no DNA unwinding activity. In the fraction III from the PIF1 overproducing strain, the 41-mer oligonucleotide was displaced when the PIF1 containing reaction mixture was incubated at 37°C. The unwinding reaction was strictly dependent on the presence of Mg^{2+} and ATP (Figure 8A, lane c). The DNA helicase activity attributed to the PIF1 protein was further confirmed by antibody inhibition studies (Figure 8B). The observed helicase activity was inhibited by the anti-PIF1 antibodies (lanes d and e) whereas it was not by the preimmune serum (f and g). SV40 T Antigen used as a positive control was not inhibited by the anti-PIF1 antibodies (lanes i and j).

PIF1 unwinds in the 5′→3′ direction

Most helicases need a free ss DNA tail to initiate unwinding and move unidirectionally with respect to the strand of DNA to which they preferentially bind. This means that there is a polarity in which the helicases unwind double-stranded DNA. To determine whether PIF1 protein translocates unidirectionally, we constructed a pair of linearized substrates (substrates II and III), with either a 3′- or 5′- ss tail. Helicase I was used as a control for unwinding in the 5′→3′ direction and T Antigen for the 3′→5′ direction. As shown in Figure 9, PIF1 unwinds only in a 5′ to 3′ direction.

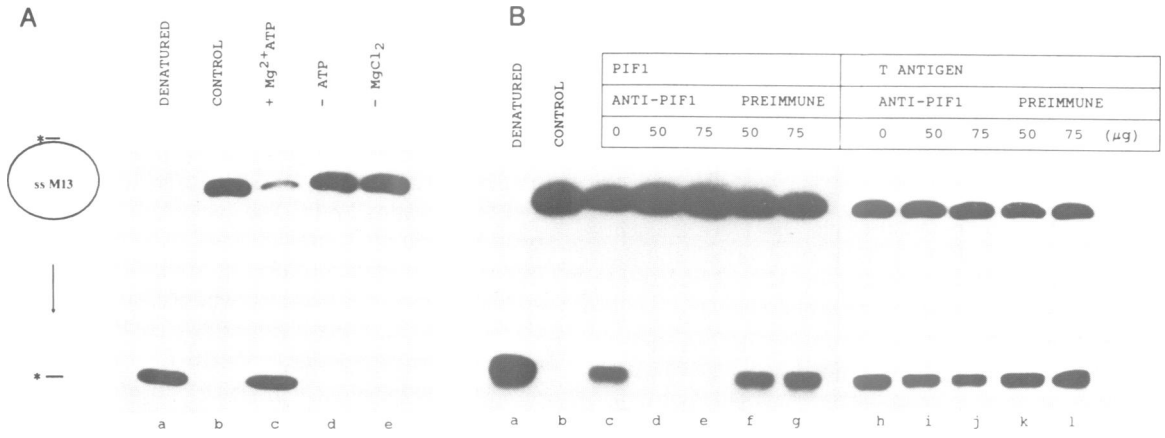

Fig. 8. PIF1 is an ATP-dependent DNA helicase. The partially duplex DNA (substrate I) used for the helicase assays consists of a 41-mer-primed M13mp7 DNA labeled with [α-^{32}P]dATP as reported in Materials and methods. Unwinding of the oligonucleotide is visualized after separation by 10% SDS−polyacrylamide gel electrophoresis and autoradiography. A schematic drawing of the substrate and product is shown on the left. **A.** Helicase activity of the PIF1 protein. Lane a, heat denatured substrate; lane b, DNA substrate I without proteins; lane c, unwinding reaction with 20 μl of fraction III in the presence of 5 mM ATP and 7 mM $MgCl_2$; lane d, as lane c but without ATP; lane e, as lane c but without $MgCl_2$. **B.** Inhibition of the helicase activity by anti-PIF1 antibodies. Reactions were performed using the same conditions as for ss DNA-dependent ATPase activity (see Figure 7). Lane a, heat denatured substrate; lane b, DNA substrate I without proteins. Fraction III (10 μl) helicase activity (lanes c−g) : lane c, unwinding reaction; lane d, as lane c but 50 μg anti-PIF1 IgG added; lane e, as lane c but 75 μg anti-PIF1 IgG added; lane f, as lane c but 50 μg preimmune IgG added; lane g, as lane c but 75 μg preimmune IgG added; SV40 T antigen helicase activity (lanes h−l) : same assays performed as with fraction III but using 300 ng of purified T antigen as a control.

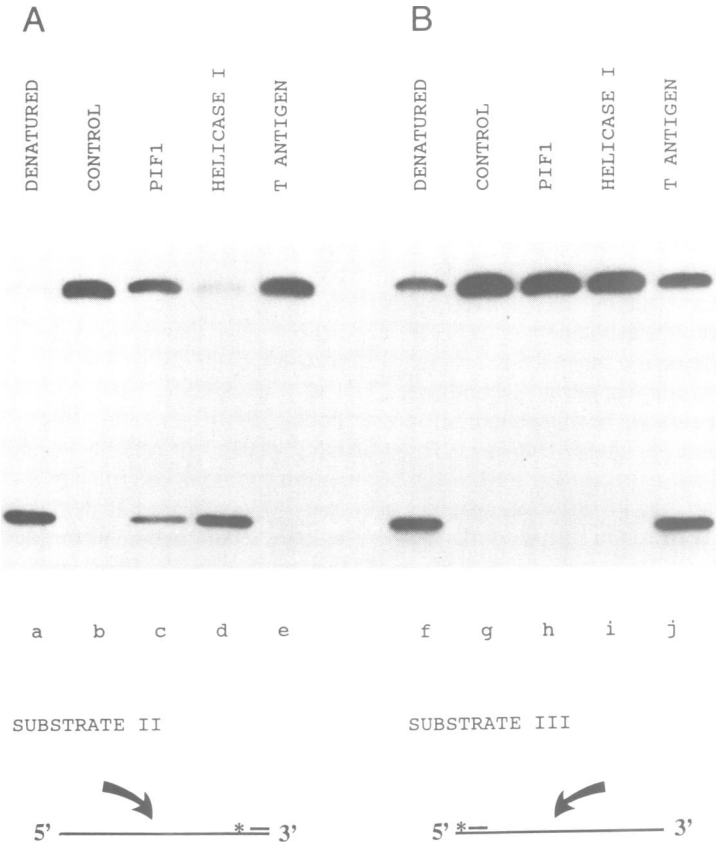

Fig. 9. Direction of unwinding. A schematic drawing of the substrates (II and III) is shown at the bottom. Linearized M13mp7 was primed at the 5′ or the 3′ end with a complementary oligonucleotide and labeled with [α-^{32}P]dATP as described in Materials and methods. **A.** reactions with substrate II. Lane a, heat denatured substrate; lane b, DNA substrate without proteins; lane c, 10 μl of PIF1-containing fraction III; lane e, 30 ng of helicase I; lane f, 300 ng of T Antigen. **B.** same as in A, except that substrate III was used in the assay.

Discussion

We have demonstrated that PIF1 is a mitochondrial DNA helicase. To our knowledge, PIF1 is the first DNA helicase to be found and characterized in mitochondria.

Strategy

Although it was shown twenty years ago that repair of yeast mitochondrial DNA occurs (Moustacchi, 1971; Moustacchi *et al.*, 1976), nothing is known about the proteins and the genes which are involved in this process. A standard approach to this problem combines genetics and biochemistry. The wild type gene (of interest) is first cloned by complementation of a deficiency mutant and sequenced. Then, the gene product is identified and purified, and with the help of sequence analysis data, its unknown function is determined by *in vitro* assays. The easy identification by Western blotting of the unknown protein requires that antibodies against the artificially expressed gene product are raised. However, in many instances, because proteins involved in DNA metabolism are often rare, the unknown protein is not immunodetected in wild type extracts. This was the case, in yeast, for the RAD3 DNA helicase required for the excision repair of nuclear DNA (Sung *et al.*, 1987a,b), or for the CBP1 protein required for the stability of the mitochondrial cytochrome *b* pre-mRNA (Weber and Dieckmann, 1990). Similarly, in our study, the absence of immunodetection of the PIF1 product in wild type mitochondria is not surprising, as predicted by the low transcription level and the codon bias index close to zero. The protein had to be overproduced by placing the *PIF1* gene under the control of the strong inducible GAL1 promoter. After galactose induction and Coomassie blue staining of a SDS−polyacrylamide gel, PIF1 was vizualized as a major polypeptide of the expected size.

Mitochondrial location and biological activity of overproduced PIF1

The overproduced PIF1 protein is biologically active. As expected, it is targeted to mitochondria as shown by several lines of evidence. First, immunoelectron microscopy reveals the presence of gold particles in mitochondria. Second, in *mas1* mutants deficient in the maturation of mitochondrially-imported proteins (Yaffe and Schatz, 1984), PIF1 accumulates as a precursor which is processed into lower molecular weight form(s) in the *MAS1* background. Moreover, the overproduced PIF1 protein restores mtDNA recombination in *pif1* mutants.

PIF1 overexpression is toxic to yeast cells. This is not an unusual feature of overproduced proteins involved in DNA metabolism. Moreover, mitochondria are essential to cell life and accumulation of PIF1 as 'inclusion bodies' (see below) might deeply disturb the mitochondrial metabolism. It must be noted, however, that overproduced PIF1 has no dramatic effect on mitochondrial DNA metabolism since no increase in the production of rho$^-$ mutants was observed. Toxicity might be partially explained by the presence of the overproduced PIF1 in the nucleus of several cells (unpublished) where it might disorganize the nuclear DNA metabolism.

Aggregation of overproduced PIF1

The overproduced PIF1 protein is highly insoluble and is only solubilized by zwittergents or denaturant agents such as urea. Overproduction often leads to aggregation in the form of 'inclusion bodies', as first shown in *E.coli* upon heterologous overexpression (reviewed by Marston and Hartley, 1990), and also occurs in yeast as observed with the overproduced proinsulin found in the cytoplasm as phase-bright inclusions (Cousens *et al.*, 1987). The overproduced mitochondrial CBP1 protein is also highly insoluble (Weber and Dieckmann, 1990). We could assume that most of the overproduced PIF1 product is not taken in charge by protein helpers like disulfide-isomerase, proline *cis−trans* isomerase and heat-shock 'chaperones' which catalyze protein folding (Pelham, 1986; Lang *et al.*, 1987; Bulleid and Freedman, 1988; Deshaies *et al.*, 1988; Chirico *et al.*, 1988; Zimmermann *et al.*, 1988; Ostermann *et al.*, 1989) and that wrong hydrophobic interactions and intermolecular disulfide bridges formation could occur, thus, eliciting the production of aggregated proteins as 'inclusion bodies'. Indeed, electron microscopy shows the presence of looking-like 'inclusion bodies' in the mitochondria of PIF1-overexpresssing transformants which are not observed in the wild type strains. HSP60 and HSP70 are present in mitochondria and are involved in folding and assembly of mitochondrial proteins (Ostermann *et al.*, 1989; Kang *et al.*, 1990). It was reported that *hsp60* mutants accumulate insoluble aggregates of mitochondrial precursors (Ostermann *et al.*, 1989) and we could hypothesize that the presence of limiting amounts of HSP60 leads to incomplete folding of the overproduced PIF1 protein. On the other hand, by comparison with the insoluble nuclear scaffold proteins like p68 RNA helicase (Hirling *et al.*, 1989), the native PIF1 protein could also belong to a 'mitochondrial scaffold', if existing, and be insoluble. Further work of purification of the native protein is required to answer this question.

PIF1 is a DNA helicase

Our search for the enzymatic activity of PIF1 has been eased by sequence analysis data which have revealed that the primary structure of PIF1 is related to that of several helicases and that it possesses the seven consensus sequences of a large family of DNA helicases (Foury and Lahaye, 1987; Hodgman, 1988; Gorbalenya *et al.*, 1988, 1989; Foury, 1990; Seraphin, 1990). In order to assay PIF1 for DNA-dependent ATPase and helicase activities, it was first necessary to partially purify the enzyme and to remove all traces of contaminating ATPase and nuclease activities. We used a strain deficient for the endo-exonuclease NUC1 (Zassenhaus *et al.*, 1988) and we took advantage of the high insolubility of the PIF1 protein to extract ~50% of the membranes proteins with Triton X-100 before solubilization of the enzyme with urea and renaturation by progressive dialysis after a hydroxylapatite batchwise step. The PIF1-enriched fraction reveals an ATP-dependent DNA helicase activity with an associated ss DNA-dependent ATPase activity. Both activities are inhibited by anti-PIF1 antibodies. A null *pif1* mutant shows no activity in a similarly purified fraction. These results demonstrate that the PIF1 protein is a DNA helicase.

DNA helicases are ubiquitous proteins which play key roles in all reactions involving extensive or local DNA melting and removal of indesirable secondary structures. DNA helicases, therefore, are associated with replication, recombination, repair machineries. A single cell contains many DNA helicases and, for example, to date, nine DNA

helicases have been identified in *E.coli* (reviewed by Matson and Kaiser-Rogers, 1990). Their importance in cell metabolism is increasingly recognized. However, their multiplicity has often hampered the identification of their specific biological function, even in *E.coli* where they are best characterized. Moreover, many DNA helicases appear to be multifunctional. The Rep protein in *E.coli* might be involved in both repair and replication; UvrD helicase plays functions in both the excision step of the nucleotide excision repair catalyzed by the UvrABC complex and in mutagenesis; SV40 T antigen unwinds DNA at the replication origin in the initiation of viral DNA replication, is used as a DNA helicase in the elongation process. Moreover, T antigen seems also to be involved in DNA recombination and possesses an RNA helicase activity (Schiedner *et al.*, 1990; Scheffner *et al.*, 1989). In contrast, helicases might also have a unique specific role like DNA helicases associated with the primosome. The DNA helicase activity of the PIF1 protein is well related with our previous genetic data showing that *pif1* mutants are deficient in mtDNA repair and in a specific mechanism of the mtDNA recombination (Foury and Kolodynski, 1983). We have shown that PIF1 stimulates the recombination between rho$^+$ and tandemly-arrayed rho$^-$ genomes through the recognition of a recombinogenic signal; this signal is not sequence specific (Foury and Van Dyck, 1985). In the 21S rRNA gene, we have identified a PIF1-dependent recombinogenic signal characterized by a 45 bp A+T sequence including a perfect palindrome over 26 bp; this sequence is certainly prone to forming secondary structures such as cruciforms that may be involved in the initiation of recombination provided that they can be melted out (Foury and Van Dyck, 1985). PIF1 DNA helicase may perfectly well fill this role.

It was shown fifteen years ago that excision repair of pyrimidine dimers induced by UV light does not exist in yeast mitochondria and it was suggested that repair occurs through recombination (Waters and Moustacchi, 1974; Prakash, 1975). It is therefore likely that PIF1 is essential to repair by its recombinational functions.

It is clear that PIF1 is not essential to the replication of mitochondrial DNA (Foury and Lahaye, 1987). However, in certain genetic backgrounds, null *pif1* mutants accumulate 20–50% rho$^-$ mutants at 28°C and completely lose mitochondrial DNA at 37°C (unpublished). This discovery suggests that PIF1 may participate in the replication process, possibly by destroying local secondary structure in a manner analogous to that of the gene 41 helicase of bacteriophage T4 (Jongeneel *et al.*, 1984).

PIF1 is the first gene product involved in repair and recombination of mitochondrial DNA whose enzymatic function has been characterized. It has a central key function. It can be assumed, therefore, that many gene products interfere with *PIF1*. It should be possible in the future to isolate new genes which may, even partially, complement null *pif1* mutants and to discover new products participating in fundamental and original mechanisms of mitochondrial DNA metabolism.

Materials and methods

Strains and media

The *S.cerevisiae* strains are listed in Table I. The following media were used. YD (2% glucose, 2% yeast extract Kat), YETOH (2% ethanol, 2% yeast extract) YGAL (2% galactose, 2% yeast extract), YG(oli) (2% glycerol, 1% yeast extract, 50 mM sodium phosphate, pH 6.5, 3 mg/l oligomycin), SD (2% glucose, 0.7% yeast nitrogen base), SETOH (2% ethanol, 0.1% glucose, 0.7% yeast nitrogen base), SGAL (2% galactose, 0.7% yeast nitrogen base). S media were supplemented with the required amino acids.

Table I. Genotypes and sources of *S.cerevisiae* strains

Strain	Genotype	Source
W303-1B	α, *ura3-1 leu2-3,112 ade2-1 trp1-1 his3-11,15 can1-100*	R.Rothstein[a]
W303-1B/PGD	as W303-1B but *pif1::LEU2*	Foury and Lahaye (1987)
A12	α, *ura3-1 trp1-1 nuc1::LEU2*	Zassenhaus[b]
A25	α, *ura3-1 ade2-1 trp1-1 nuc1::LEU2*	This study[c]
A25/PGD	as A12 but *pif1::URA3*	This study[d]
JF2-2B/1-41	a, *his pif1-1* rho$^-$ O^R_{625}	Foury and Kolodynski (1983)
AH216-1A	a, *leu2-3,112 his3-11,15 phoE phoC*	Yaffe and Schatz (1984)
MY11-D72	as AH216-1A but ts *mas1*	Yaffe and Schatz (1984)

[a] R.Rothstein, Columbia University, New York, USA
[b] H.P.Zassenhaus, St Louis University Medical Center, St Louis, USA
[c] A25 is a meiotic segregant issued from the cross A12×W303-1B
[d] In A25/PGD the chromosomal *PIF1* gene was disrupted by deletion of a 29 bp fragment and replacement by a 1.2 kb *Bgl*II fragment containing the *URA3* gene isolated from the pFL44 vector (F.Lacroute, Gif-sur-Yvette, France)

Production of anti-pif1 antibodies

In order to produce a PIF1-β-galactosidase fusion protein in large quantities, the *PIF1* gene was placed under the control of the strong P_R promoter of bacteriophage lambda and the thermosensitive CI_t repressor in the vector pCQV2 (Queen, 1983). The N-terminal region of the *PIF1* gene deleted of its first 96 bases was inserted downstream of the P_R promoter in frame with the AUG codon of the *CRO* gene while the C-terminus was fused in frame with the *lacZ* gene. After induction at 42°C, the fusion protein containing ~93 kd of the PIF1 protein was recovered in an insoluble fraction and used for antibody preparation essentially as described by Tjan *et al.* (1974). The fusion protein was purified by SDS–polyacrylamide gel electrophoresis. After gel staining with Coomassie blue for 30 min and destaining overnight in 30% methanol, 10% acetic acid, the gel slice containing the fusion protein was cut out, washed with a phosphate-buffered saline (PBS, 8 mM sodium phosphate, pH 7.4, 100 mM NaCl) and lyophilized. A goat was used for immunization. Immunization consisted in three injections at one week intervals, followed by a fourth injection one month later. Lyophilized acrylamide was suspended in 0.9% NaCl. For the primary injection, ~100 μg of fusion protein was emulsified with 1 ml of Freund's complete adjuvant (1:1). For the other injections, ~50 μg of fusion protein was mixed with an equal vol of incomplete adjuvant.

Overproduction of the PIF1 protein in yeast

The *PIF1* gene was fused to the inducible yeast GALI (galactokinase I) promoter in the vector pLGALZ3, which contains the yeast 2 μ origin of replication, *URA3* and *LEU2D* as selection markers. The resulting plasmid pLGALP38 contains the *PIF1* gene and flanking non-coding regions from position −80 upstream of the AUG initiator codon and up to 500 nucleotides downstream of the *PIF1* stop codon (Figure 1) (the detailed construction of the yeast expression vectors will be published elsewhere).

Galactose induction of the *PIF1* gene expression was achieved as follows. Yeast cells were inoculated from a 24 h SD preculture into a YETOH medium at a cell density of 8×10^6 cells/ml. After 10 h of growth, galactose was added to the culture to a final concentration of 2% and growth was further proceeded for 14 h before harvesting the cells.

Immunoelectron microscopy

For immunoelectron microscopy studies, immunoglobulins (IgGs) were purified by the 'caprylic acid' method (McKinney and Parkinson, 1987). Quantity of IgG was determined by the formula [IgG] = $OD_{280\ nm}/1.43$.

Immunoelectron microscopy experiments were carried out essentially as described by Jacobs *et al.* (1989). Galactose grown cells were directly fixed in 3% formaldehyde and 0.25% glutaraldehyde, treated with sodium metaperiodate and embedded in Epon. Ultrathin sections (300 Å) were cut with an Ultratome III (LKB) equipped with a diamond knife (Diatome) and mounted on 400-mesh Ni-grids. Immunodecoration was performed by using 1.2 mg/ml purified IgG from the preimmune or the anti-PIF1-containing serum and gold-labeled protein A (Auro Probe EM, Janssen Life Sciences Products).

PIF1-dependent recombination phenotype

PIF-dependent oligomycin resistant (O^R) rho^- and rho^+ strains of opposite mating type were mated on YD (or YGAL, when induction of the *PIF1* gene expression from the pLGALP38 yeast vector was needed). After one day of incubation at 30°C the prototrophic diploids were selected on minimal glucose (SD) medium. After one more day of incubation at 30°C the rho^+ strains that integrated the oligomycin resistant marker were selected on YG(oli) medium. High frequency of recombination promoted by the *PIF1* gene was observed when rho^+ diploids gave confluent growth on oligomycin medium.

Partial purification of the PIF1 protein

Mitochondria were prepared from spheroplasts as described by Daum *et al.* (1982). To prepare mitoplasts, mitochondria were osmotically shocked in 10 mM Tris−HCl, pH 7.5, 0.1 M mannitol. The resulting mitoplasts, suspended in a buffer containing 10 mM Tris−HCl, pH 7.5, 0.45 M sucrose, 2 mM ATP, 2 mM $MgCl_2$, were separated into the membrane and the matrix fractions after sonication and centrifugation. All steps were performed at 0−4°C. Mitochondrial membranes were suspended to a final protein concentration of 2 mg/ml in 50 mM Tris−HCl, pH 8.5, 1 mM EDTA, 10 mM β-mercaptoethanol (β-MSH) and 1% Triton X-100. After 30 min of incubation, the suspension was centrifuged for 30 min at 100 000 *g*. The pellet, containing the PIF1 protein, was washed with the same buffer without detergent and suspended in 6.5 M urea, 10 mM β-MSH to a protein concentration of ~ 1 mg/ml. After incubation for 1 h, the urea suspension was centrifuged for 30 min at 100 000 *g*. The urea supernatant containing the PIF1 protein was then subjected to batchwise adsorption to hydroxylapatite (Biogel HTP, Biorad) (fraction I). After incubation for 30 min with hydroxylapatite (~ 1 ml/mg of proteins) preliminary equilibrated with 6.5 M urea and 10 mM β-MSH, the suspension was centrifuged for 5 min at 1 500 *g*. The hydroxylapatite pellet was washed with 6.5 M urea, 10 mM β-MSH, 50 mM Na_2HPO_4 and PIF1 protein was eluted by two successive aliquots of 6.5 M urea, 10 mM β-MSH, 200 mM Na_2HPO_4 (fraction II). Fraction II was dialyzed first for 90 min against 100 mM Tris−HCl, pH 9.5, 2 M NaCl, 1 mM EDTA, 10 mM β-MSH, and the dialysis was further pursued for 20 h, with one change, against 25 mM Tris−HCl, pH 9.0, 100 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, 10 mM β-MSH (fraction III).

ATPase assay

The standard assay mixture (50 μl) contained 50 mM Tris−HCl, pH 8.5, 100 mM NaCl, 7 mM $MgCl_2$ and 5 mM ATP. After incubation for 30 min at 37°C, the released phosphate was measured at an optical density of 820 nm as described by Ames (1966). DNA-dependent activity was measured by using 1 μg of poly(dC) per assay and comparing it with an assay without homopolynucleotide.

Helicase assay

Three different helicase substrates were prepared using M13 mp7 DNA. For substrate I (see Figure 8), 1 μg of ss circular M13 DNA was annealed to 20 ng of the complementary 30-mer oligonucleotide 5′-CGTAATCATGGTCATAGCTCTTTCCTGTGT-3′ in 20 mM Tris−HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl and 1 mM dithiotreitol by heating the reaction mixture (20 μl) at 80°C for 10 min and allowing slow cooling to room temperature. The resulting partial duplex was extended to 41 bp at the 3′-OH end of the oligonucleotide by incubation with the Klenow fragment of DNA polymerase I in the presence of 10 μCi of [α-^{32}P]dATP (specific activity 3000 Ci/mmol), 50 μM dGTP and 50 μM dTTP for 25 min at 25°C. The mixture was incubated for an additional 15 min with 50 μM of unlabeled dATP. The reaction was ended by the addition of one volume of a buffer containing 10% SDS, 50 mM EDTA, 150 mM NaCl. Partial duplex DNA was separated from deoxynucleotides and unannealed oligonucleotides by size exclusion chromatography on Sepharose CL-4B (Pharmacia). For the two other substrates, ss M13mp7 DNA, which contains a hairpin and can be cleaved by *Eco*RI and *Bam*HI, was linearized and annealed to an oligonucleotide complementary to the 5′ or the 3′ end (see Figure 10). Substrate II was made by hybridization of the same 30-mer oligonucleotide used above to *Eco*RI linearized ss M13mp7 and extension to 41 bp just as described aboved. Substrate III was made by hybridization of the *Bam*HI linearized M13mp7 to the 15-mer universal primer 5′-TGCAGCACTGACCCT-3′ and run 'off' synthesis by the Klenow fragment in the presence of 50 μM of dCTP, dGTP and dTTP each and 10-20 μCi of [α-^{32}P]dATP resulting in radioactive extension to 46 bp. The reaction was chased in the presence of 50 μM unlabeled dATP and the DNA was isolated as described before.

The standard helicase assay mixture (50 μl) contained 1−3 ng of substrate DNA (~ $1-3\times10^3$ c.p.m.) and otherwise was as described for ATPase assay. Reactions were performed at 37°C for 30 min and were ended by addition of 5 μl of stop solution containing 30% glycerol, 150 mM EDTA and 2% SDS. Strand separation was assessed by electrophoresis in 10% SDS−polyacrylamide gels as described by Laemmli (1970) followed by autoradiography on Kodak X-Omat-AR film.

Other procedures

DNA techniques were done as described in Sambrook *et al.* (1989). Proteins were measured as described by Lowry *et al.* (1951) after precipitation by the chloroform−methanol procedure (Wessel and Flügge, 1984) to remove β-MSH which interferes with the Folin reagent. Total yeast extracts were prepared essentially as described by Yaffe and Schatz (1984). Extraction and precipitation of yeast proteins were performed by a treatment with a NaOH/β-MSH solution and trichloracetic acid; the resulting pellets were washed successively with ethanol:ether (1:1) and ether before drying and suspension in the buffer for electrophoresis analysis. Proteins were separated by electrophoresis in SDS−polyacrylamide gels as described by Laemmli (1970). Proteins were transferred to nitrocellulose membranes as described by Towbin *et al.* (1979). For Western blots, the nitrocellulose membrane was saturated by incubation in 1% Tween-20, 50 mM Tris−HCl, pH 7.5, 150 mM NaCl; 0.1% Tween-20 was used for the other steps; antibodies were used at 1:100 dilutions and reaction was visualized by ^{35}S-labeled protein A (Amersham) and autoradiography on Amersham β-max film.

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